SPATIAL TRENDS IN MERCURY EXPOSURE, ITS DIETARY SOURCES AND CLINICAL HEALTH EFFECTS IN FINNISH WHITE-TAILED EAGLE NESTLINGS

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Despite the current increasing population trend, white-tailed eagles are still prone to many anthropogenic stressors. Mercury (Hg) is a very persistent naturally occurring element and apex predators as well as birds feeding on aquatic ecosystems are usually exposed to higher levels of Hg. Since global Hg emissions are still increasing, Hg remains as a topical health issue. The effects of mercury on wildlife have been studied extensively. Blood clinical-chemical parameters (BCCPs) can potentially be useful as biomarker endpoints for contaminant exposure. However, the effects of Hg on blood chemistry and metabolism are still poorly described. Mercury concentrations and stable isotope composition can be measured from the feathers of nestlings and the nestlings represents well the geographical area of interest. Stable isotope analyses can be used to investigate the potential dietary sources of mercury.

In the experimental part we investigated the regional differences in stable isotope values, Hg concentration and BCCPs, as well as the interactions between stable isotope values and Hg exposure. We also investigated possible effects of Hg exposure on the health of WTE nestlings in three populations in Finland perceived by BCCPs. Blood and feather samples were collected from WTE nestlings in Finland during the years 2015 and 2016 in three different locations. Blood plasma was analysed for 16 different BCCPs. Feathers were analysed for total Hg concentration as well as for stable nitrogen (δ^{15}N) and carbon (δ^{13}C) composition.

A significant difference in δ^{13}C and δ^{15}N values in Varsinais-Suomi and Ostrobothnia compared to Lappi was apparent and is likely to indicate variable local dietary habits. Nonetheless, the concurrent variation in δ^{15}N and δ^{13}C values is indicative of changes in prey composition rather than regional changes in baseline isotope signatures. We noted significantly higher Hg concentrations in Lapland population compared to the other regions. Since there was a high correlation between Hg and stable isotope values, dietary habits seem to have a strong effect on Hg exposure in WTE nestlings in Finland. In addition Hg was positively associated with alanine aminotransferase and total bilirubin. The statistical relationship between Hg and total bilirubin could indicate negative impact on liver function.

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Merikotkat altistuvat edelleen useille ihmisen aiheuttamille häiriötekijöille, vaikka merikotkakanta on ollut kasvussa viimeisten vuosikymmenien aikana. Elohopea on luontaisesti ympäristössä esiintyvä erittäin pitkäikäinen raskasmetalli. Vesiekosysteemeistä ravintonsa hankkivat linnut sekä huippupedot altistuvat yleensä suuremmille elohopeapitoisuuksille. Kasvavat globaalit elohopeapäästöt aiheuttavat jatkuvan terveysuhan ympäristölle ja ihmisille maailmanlaajuisesti.

Elohopean mahdollisia terveyshaittoja linnuilla on tutkittu runsaasti. Yleisimmät haittavaikutukset niillä liittyvät lisääntymiseen. Aikaisemmissa tutkimuksissa on selvitetty myös verestä mitattujen kliiniskemiallisten veriarvojen hyödyllisyyttä elohopea-altistuksen terveyshaittojen arvioinnissa kotkilla sekä muilla linnuilla. Elohopean vaikutukset kliiniseen kemiaan ovat edelleen huonosti tunnettuja.

Aiemmat tutkimukset osoittavat, että merikotkan poikasista mitatut elohopeapitoisuudet edustavat hyvin paikallista altistumista ympäristön sekä ravinnon elohopeapitoisuuksille. Elohopeapitoisuuksia voidaan tutkia merikotkapoikasten höyhennäytteistä, jolloin ne edustavat poikasten pesäaikana kokemaa elohopea-altistusta. Ekotoksikologisissa tutkimuksissa pyritään usein myös selvittämään ravinnon mahdollisia vaikutuksia vieras-ainealtistukseen. Isotooppianalyysien avulla voidaan tutkia ravintoverkkojen rakennetta perinteisiä menetelmiä tehokkaammin. Hiili-isotooppiarvot tarjoavat tietoa ravinnon lähteestä ja typpi-isotoopit eläimen ravintoketjutasosta.

Kokeellisessa osassa tutkimme elohopean, hiili- ja typpi-isotooppien sekä kliiniskemiallisten veriarvojen alueellisia eroja sekä mahdollisia riippuvuussuhteita elohopean ja kliiniskemiallisten veriarvojen välillä. Sen lisäksi tutkimme isotooppien ja elohopea-altistuksen välisiä riippuvuussuhteita. Keräsimme merikotkan poikasista veri- ja höyhennäytteitä kolmelta eri alueelta (Varsinais-Suomi, Pohjanmaa sekä Lappi) Suomessa vuosina 2015 ja 2016. Analysoimme verestä 16 eri veriarvoa ja määritimme höyhenistä niiden elohopeapitoisuuden sekä pysyvien hiili- ja typpi-isotooppien koostumuksen.

Tutkimuksessa havaitsimme merkittäviä eroja hiili- ja typpi-isotooppien koostumuksessa merikotkan poikasissa Lapin sekä Varsinais-Suomen ja Pohjanmaahan välillä. Ero kuvastaa todennäköisesti merikotkien paikallisia ravintotottumuksia sekä eroja saaliin koostumuksessa. Havaitsimme lisäksi merkittävästi korkeampia elohopeapitoisuuksia Lapin merikotkapopulaatiossa. Ero voi johtua Lapin tekojärvien korkeammasta elohopean taustapitoisuudesta sekä elohopean kertymisestä ravintoketjussa. Havaitsimme tilastollisesti merkittävän riippuvuussuhteen elohopean ja isotooppiarvojen välillä, mikä todennäköisesti kertoo ravinnon olevan merkittävä tekijä elohopea-altistukselle merikotkan poikasilla Suomessa. Havaitsimme merkittävän positiivisen riippuvuuden elohopean ja alaniiniaminotransferaasin sekä kokonaisbilirubiinin välillä. Tulos voi viitata mahdollisiin haittavaikutuksiin maksassa kokonaisbilirubiinin osalta.

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1 INTRODUCTION

The White-tailed eagle (*Haliaeetus albicilla*) is a species well known for being a conservation icon due to surviving drastic population declines caused by chemical pollutants, such as dichlorodiphenyldichloroethylene (DDT), polychorinated biphenyls (BCP's) and mercury (Hg) in Europe between 1950s and 1980s. Due to the bans and restricted use of these chemicals the reproduction success started to increase again slowly (Helander et al. 2002) and good environmental status (GES), which is evaluated by estimated target levels for brood size, breeding success and productivity, has been mostly achieved in the Baltic coast except for the Swedish coast of the Gulf of Bothnia and the Latvian coast. Despite the current increasing trend in population numbers, White-tailed eagles (WTE) are still prone to many anthropogenic stressors which may affect their productivity by decreasing breeding success and nestling brood size (HELCOM 2015). Population development models have predicted that certain stressors might restrict the WTE population growth locally (Korsman et al. 2011). Due to these potential negative effects on population development, the species still needs attention by both researchers and stakeholders.

Mercury (Hg) is a very persistent naturally occurring element, which has a notorious reputation due to high levels of anthropogenic use and emissions before the restrictions and bans on use in Europe. Despite legislative efforts and locally decreased emissions in Europe, Hg emissions have increased on a global scale to date. These emissions reach Europe due to the global nature of Hg pollution (e.g. air transport) and it is estimated that 50% of the anthropogenic Hg deposited annually in Europe originates from outside of Europe (EEA 2018). Apex predators, such as birds of prey are usually exposed to higher levels of these persistent contaminants due to the process of biomagnification, where a compound increases its concentration in the organism as it travels up the food chain, which has been well-documented for aquatic food webs (Furness 1993, Morel et al. 1998). Moreover, it has been suggested that exposure to elevated Hg concentrations is more significant in young individuals (Wolfe et al. 1998). However, Hg levels occurring in natural prey items rarely exceed the limit of lethal dose (Henny et al. 2002, Cristol et al. 2008, Miller et al. 2012). Studies investigating sublethal effects of Hg have therefore increased, though effects on blood chemistry and metabolism are still poorly described (Whitney & Cristol 2018).

Blood clinical-chemical parameters (BCCPs) can potentially be useful as biomarker endpoints for contaminant exposure (Sonne et al. 2012), and birds of prey are good sentinels for environmental pollution due to their top position in the food chain (Burger 1993). Since the

WTE's conservation status falls currently in the category of least concern, it is relatively easy to obtain study material from this particular species (Birdlife international 2016). The use of nestlings for biomonitoring has certain advantages because they represent well the geographical area of interest (Olsson et al. 2002). Moreover, possible confounding factors, such as migration and reproductive status can be excluded (Eulaers et al. 2011). Studies investigating Hg exposure and its possible effects on bald eagle (*Haliaeetus leucocephalus*), a species considered to be a close relative of the WTE, have been studied extensively in North America. One study found significantly elevated Hg concentrations, approaching sublethal levels, in inland bald eagle population compared to coastal ones (Kramar et al. 2019). Similar studies comparing Hg exposure in different eagle populations have not been done in Finland.

In the present thesis we therefore study three WTE subpopulations, in Southern Finland, Ostrobothnia and Lapland. The first objective of the present study investigates the spatial variation of Hg exposure in these different subpopulations. The second objective uses dietary tracers, i.e. stable nitrogen and carbon isotopes, to evaluate how trophic position and differences in food chain carbon source may affect Hg exposure. The third objective investigates potential clinical effects of Hg, with a special focus on spatial differences.

2 LITERATURE REVIEW

2.1 Mercury

Hg is a naturally occurring soil element that is ubiquitously distributed in the Earth's crust and it is known to be the only metal that is in liquid form in the room temperature. It is rarely found as a native metal, but more usually as a mineral compound, such as cinnabar and livingstonite (Rytuba 2003). The vast anthropogenic use, for example in the industry, has led to its global release into the environment (EEA 2018). The use of Hg has caused detrimental events in the history, such as methylmercury (MeHg) poisoning at Minamata bay in 1956 (Kudo & Miyahara 1991). Hg poisonings were also later recorded in Europe and North America in the birds of prey, especially within WTEs in Europe (Henriksson et al. 1966, Helander et al. 1982, Scheuhammer et al. 2007). This was due to the agricultural use of Hg containing pesticides, which eventually led to the bioaccumulation and biomagnification of Hg in the food webs (Scheuhammer et al. 2007). Despite the restrictions and bans on the use of Hg and its compounds have been placed in EU, the global use of Hg has increased, especially in Asian countries, mostly due to small-scale gold mining and vinyl chloride manufacturing (VCM) (EEA 2018). The Minamata convention on Hg, which was adopted and signed in 2013, will

restrict the use and enhance the reduction in Hg pollution globally for activities which are mainly accountable for Hg emissions (UNEP 2013). It is estimated that the concentrations of Hg in the environment will yet increase and that the climate change can have exacerbating effects on it (Stern et al. 2012). For example, increased incidence in wood fires, rainfall and flooding can promote emissions and the distribution of Hg (Amos et al. 2013). In addition, melting permafrost may expose new Hg sources (Pierre et al. 2018). In order to stabilize background concentrations, strict control on emissions are needed (Krabbenhoft & Sunderland 2013).

2.1.1 The global mercury cycle

Hg exists in three different chemical forms in the environment which differ by their toxicological and biochemical properties. These different forms of Hg are categorized as inorganic, organic and elemental Hg. The most common and toxic form of organic Hg is MeHg (Ramesh 2012). It is mainly found in the aquatic environment (inland waters, marine environment) because some anaerobic sulphate- and iron-reducing bacteria are able to convert Hg into MeHg (Podar et al. 2015). Additionally, some specific abiotic factors in the environment may enhance the bioavailability of MeHg. Some of the known factors are acidification and high dissolved organic carbon concentrations (Driscoll et al. 2007). Inorganic Hg compounds are powders or crystals, where Hg occurs as salts. The primary inorganic form of Hg is mercuric chloride (Fisher 2003). Elemental Hg is rare in its pure form and it is mainly used in anthropogenic activities. Hg is also easily released into the atmosphere, due to its high vaporizing properties (Rytuba 2003).

Along with anthropogenic activities, Hg is distributed throughout the environment by natural processes, such as volcanic activity, oceanic upwellings, movement of rivers and fires (Driscoll et al. 2013). Nowadays Hg emissions by anthropogenic activities are more significant for the distribution of the substance compared to natural processes (EEA 2018). Together, these anthropogenic and natural processes drive the biogeochemical flux of long-lived Hg reservoirs from earth crust into atmosphere and freshwater, marine and terrestrial environments (Driscoll et al. 2013). The transportation of Hg occurs by atmosphere, water and through food-chains (Fisher 2003). The atmosphere is the most important pathway for Hg transportation (Schroeder & Munthe 1998). Hg enters the atmosphere by vaporization from the soil or through anthropogenic emissions and natural processes such as wood burns and volcanic eruptions (Nriagu & Becker 2003, Friedli et al. 2009). In the atmosphere over 90% of the Hg is in the elemental form, and hence it is volatile, it may persist in the atmosphere for several months to a year (Schroeder & Munthe 1998). In the atmosphere Hg can be driven to distant locations

(Dunford et al. 2010) before deposition on to soil or water by rainfall or other weather conditions or by direct binding. In the water elemental Hg either re-enters the atmosphere or forms inorganic compounds (Fisher 2003). Inorganic Hg compounds are then converted in to organic Hg compounds, such as MeHg, by biotransformation in sediments and within organism by some anaerobic bacteria (Podar et al. 2015). Since MeHg has a high affinity towards thiol groups in proteins (Harris et al. 2003), it remains in both muscles and fatty tissues, which further enhances its' bioaccumulation and biomagnification properties (Watras & Bloom 1992). Hg further accumulates through food chains in the organisms and elevation in Hg concentrations are usually related to higher trophic levels (Atwell et al. 1998). Hg can end up in the water also due to waste water discharge and land erosion or flooding (Hargreaves et al. 2016, Kwasigroch et al. 2018).

2.2 Toxicokinetics of Hg

2.2.1 Exposure

Hg is a non-essential metal for mammals with potential neurotoxic properties. The major route for exposure of Hg is through ingestion (Monteiro & Furness 1995). In addition, exposure to different forms of Hg by dermal absorption and inhalation is possible, although of little importance (Ramesh 2012). Maternal transfer of Hg can act as an important route of exposure for embryos and nestlings (Ackerman et al. 2016) and it has been proven that hatching order may also affect the level of exposure (Becker 1992). Hg exposure in nestling is also dependent on chick age and food ingestion (Becker et al. 1994, Monteiro & Furness 1995).

Feeding habits and trophic position may influence variation in Hg concentration in birds (Provencher et al. 2014). Birds that consume freshwater and marine organisms are thought to exposure to higher levels of Hg compared to species which feed on terrestrial organisms (Solonen & Lodenius 1990). However, recent studies have investigated that exposure to Hg is not only limited to species feeding in aquatic environment but occurs also in terrestrial species by a trophic transfer between contaminated aquatic bodies, such as rivers, and terrestrial habitats (Howie et al. 2018). Other factors which can affect to the magnitude of exposure in addition to trophic level, include regional and geographical differences in Hg availability (Moreno et al. 2011), individual traits such as age, sex, body size and lifespan (Evers 1998, Bearhop et al. 2000, French et al. 2010), moulting pattern, habitat, migratory habits (Monteiro & Furness 1995, Klaassen et al. 2012) as well as interspecies and intraspecies variation in diet preference (Bearhop et al. 2000).

2.2.2 Absorption, distribution and metabolism

The absorption of Hg is highly dependent on the form in which it is ingested. Metallic Hg is absorbed only a little through gastrointestinal tract, but due to its lipophilicity, it can cross blood-brain barrier. In the brain, metallic Hg can be converted into inorganic divalent cation Hg, which cannot pass the blood-brain barrier and can therefore persist in the brain for an extended period of time (Ramesh 2012). There is a growing body of evidence on Hg neurotoxicity in avian models (Whitney & Cristol 2018). Possible impairments include axonal degeneration (Bennett et al. 2009) as well as hearing impairment (Wolf et al. 2017).

The absorption of ingested inorganic Hg in mammals differs between 10-40%, whereas the absorption of ingested organic Hg compounds is almost perfect (Ramesh 2012). The absorption rate of MeHg from the gastrointestinal tract after ingestion has been estimated to be 83% in common loons (*Gavia immer*) (Fournier et al. 2002). This is slightly less compared to the reported absorption rate in mammals, which is around 90-95% (Ramesh 2012). Absorbed MeHg is further transported into the circulation through liver although some reabsorption of MeHg occurs in the gut due to enterohepatic circulation (Norseth & Clarkson 1971, Norseth 1973, Ballatori & Clarkson 1982). Most of the MeHg in the blood is bound to haemoglobin within red blood cells. The rest of MeHg is bound to plasma proteins and is furthermore distributed relatively rapidly into other tissues and organs until it can be further eliminated (Nichols et al. 2010). In birds Hg tends to accumulate in the kidneys, liver, muscle and brain (Kalisinska et al. 2014) although species and age-related differences in distribution patterns exists (Hopkins et al. 2007). MeHg is transferred also readily in keratin structures such as feathers (Monteiro & Furness 1995) and into developing eggs (Hammerschmidt et al. 1999, Evers et al. 2003).

2.2.3 Elimination

The biological half-life of the Hg depends on many factors. Usually metallic Hg accumulates in the kidneys and is eliminated slowly (Ramesh 2012). In birds, elimination of mercury occurs through droppings, feather moulting, egg laying and secretion through uropygial or salt glands (Thompson & Furness 1989, Lewis & Furness 1993, Dmowski 1999). Significant hepatic demethylation of MeHg is known to happen in some seabirds. This is thought to be an adaptive response to the dietary content (Norheim 1978, Nigro & Leonzio 1996, Thompson & Furness 1989). Furthermore, elimination processes are distinct to different species and hence must be investigated through experimental studies. It is shown for example in Japanese quail (*Coturnix japonica*), that excretion in droppings plays a dominant route for MeHg elimination, which is the primary route for mammals as well (Lewis & Furness 1993, Ramesh 2012). The ratio of

elimination between different routes may differ in male and female sexes due to egg laying and must be taken into consideration since it has a large impact on the circulating MeHg concentration (Becker 1992, French et al. 2010). For example, one study found that female herring gull (*Larus argentatus*) eliminates nearly 20% more of their body burden compared to males due to egg laying (Lewis et al. 1993). Other important factors influencing the elimination rate includes migration (Seewagen et al. 2016), feather moulting patterns (Braune 1987) and age. Elimination half-time have been observed to be shorter in chicks compared to adults, which probably depicts the prompt metabolism and growth of tissues in younger individuals (Furness & Monteiro 2001b). Also, body size has been identified as an important factor for Hg concentrations in blood. According to Ackerman et al. (2011) Hg concentration declined more in those chicks that gained proportionately more mass between the sampling events. Elimination of MeHg into feathers in birds is also significant, and the magnitude usually differs between flight-, body-, and tail feathers due to their difference in size and growing pattern (Braune 1987, Lewis & Furness 1991).

2.3 Hg toxicity thresholds and endpoints in Birds

As with all toxic substances, Hg's effects are dose related and the effects of Hg on wildlife have been studied extensively (Scheuhammer et al. 2007). To assess the risk of exposure the threshold levels need to be identified. These threshold levels are usually characterized by no-observed-adverse-effect level (NOAEL) and lowest-observed-adverse-effect level (LOAEL). These values are determined by estimation, laboratory studies or detection of adverse effects (Ramesh 2012). Using lethal dose (LD50) point estimate is another way to evaluate toxic thresholds for mercury (Evers 2018). However, these methods may be problematic to use in the field due to the fact, that natural settings are hard to imitate. Therefore, laboratory settings tend to underestimate external stressors, which seems to be important for the response on elevated MeHg levels in the wild (Hallinger & Cristol 2011). It is suggested, that the combination of relevant controlled laboratory experiments and field studies would create more confident results for estimating adverse effect thresholds (Evers 2018).

Critical level of 5 mg/kg in feathers have been associated with reduced hatching rate and decreased chick survival, as well as lower clutch and egg size (Eisler 1987). Burger & Gochfeld (1997) proved also that 5-40µg/g dry weight (dw) in feathers would indicate for adverse health effects. However, not all species suffers even from high levels of contamination, since negative effects on reproduction parameters was not noted in Bald eagles, with feather Hg concentration of 66 mg/kg (Bowerman et al. 1994). Hence it can be argued that effect levels are usually species specific, and general threshold levels are difficult to establish. Moreover,

Hg levels occurring in natural prey items rarely exceeds the reported lethal doses for birds (Cristol et al. 2008, Miller et al. 2012) and therefore experimental as well as field studies investigating sublethal exposure to Hg have been increasing. Examples of possible sublethal health effects of Hg exposure includes end-points in behaviour, endocrine function, growth, reproduction, immunology, oxidative stress and neurology. The most common studied endpoint is related to bird reproduction and examples of adverse effect of Hg on bird reproduction includes reduced hatching rates and decreased clutch size (Hallinger & Cristol 2011, Tartu et al. 2013) Although multiple studies exist comprising of estimating the associations of Hg with different end-points, these studies can usually find only correlative relationships. Thus, it is difficult to interpret dose-response relationships, especially because field studies usually fail to differentiate the possible confounding effects and on-going environmental variables (Nichols et al. 2010).

2.4 The use of feathers for evaluating Hg contamination

Different tissues have specific Hg turnover rate, therefore representing different temporal scales on Hg exposure. Disposition of Hg into the disulphide rich keratin structures of the plumage occurs during the feather growth and reflects the exposure over time frame of approximately 1-3 weeks. Hg is transported into the feather through blood supply, which gradually withers as the feather matures (Burger 1993). The relationships among tissue types is important to comparison species-specific pharmacokinetics as well as exposure and possible affected end-points of mercury reported in the literature (Evers 2018). According to Furness et al. (1986) the MeHg concentration in the feathers has a direct relationship with the body burden of MeHg in nestlings, hence feathers may be used in order to evaluate the total exposure of Hg in the body. Monteiro and Furness (2001b) also found a positive linear relationship between body feathers and blood concentration in seabird nestlings.

Feathers can also be used to study the external contamination of heavy metals e.g., through dust. Hence this needs to be taken into consideration when preparing the feathers for chemical analyses (Furness 1993, Dauwe et al. 2003) although it has been proven that Hg in feathers is mainly due to endogenous deposition (Veerle et al. 2004). Total Hg concentration, including inorganic Hg as well as MeHg, is usually used as a proxy when measuring the contamination levels, although most of the Hg contained in feathers occurs in the form of MeHg (Bond & Diamond 2009, Bond et al. 2015). Feathers serve also as a minimally invasive method to study the contaminant exposure, since usually only a small amount of feathers are needed for the studies and sampling can be done without harming the bird (Eulaers et al. 2011a). Birds of prey nestlings are considered to represent local environmental contamination and hence the

use of nestlings for sampling may help to overcome some confounding factors, such as migration and reproduction status (Eulaers et al. 2011a).

2.5 Blood clinical-chemical parameters

Blood clinical-chemical parameter (BCCP's) analysis is used as a non-destructive method of evaluating the health and function of different organs and physiological functions in living animals. Different biomarkers reflect particular organs or bodily functions and the evaluation should be based on the knowledge of the normal physiological mechanisms and reference values in particular species in order to recognize diseases or malfunctions (Thrall et al. 2004). Generally increased or decreased concentration of a particular parameter is perceived as a pathological condition, in accordance with the clinical picture (Whalan 2015). Evaluation of BCCP values is always based on reference intervals. Reference intervals are typically determined by calculating the upper and lower limits of analytes and using the central 95% of the seemingly healthy population values, while cutting the extreme outlying values off (Geffré et al. 2009, Katayev et al. 2010). Reference values vary greatly between different laboratories due to differences in machines, reagents and the methods used. This is why every laboratory should create their own species- and methodology specific intervals (Solberg 1988, Friedrichs et al. 2012). There are also multiple factors related to sampling which might influence the results and therefore adequate handling of the sample is essential to attain high-quality results (Gilor & Gilor 2011). These includes under- or overfilling of a blood collection tube, causing erroneous anticoagulant to blood ratios. Also, haemolysis (breakage of erythrocytes) should be avoided by proper handling of the sample, because it directly interferes with spectrophotometric absorbance readings (Meyer & Burkhard 1992). Haemolysis may cause falsely high aspartate aminotransferase (AST) as well as potassium levels (Harrison & Lighfoot 2006). Lipemia is another common interference causing artefacts to blood samples due to accumulation of lipoprotein particles in plasma. It can occur in postprandial samples (Nikolac 2014) and it causes errors in many spectrophotometric and in all refractometric methods. Lipemia has been reported to cause falsely increased liver enzymes as well as elevated haemoglobin, glucose, calcium and phosphorous levels (Harrison & Lighfoot 2006). There are techniques that can resolve excess lipoprotein particles, but they must be used appropriately (Nikolac 2014). Some parameters differ also accordingly to individuals age. For example, alkaline phosphatase is generally higher in growing animals due to osteoblast activity (Harrison & Lighfoot 2005). Young animals usually have increased levels of growth hormone and mobilization activity which can cause higher phosphorus and potassium levels (Clubb et al. 1991).

BCCPs might act as useful biomarkers for evaluating contaminant exposure but are still quite rarely used in ecotoxicological studies (Stout et al. 2010, Sonne et al. 2012). Reference values for haematology and blood clinical chemistry have been described for wild Bald Eagle nestlings (*Haliateeus leucocephalus*) (Bowerman et al. 2000, Mealey et al. 2004). However comparable interpretation should be used cautiously, since BCCPs are usually species specific. Furthermore, biological variation and environmental conditions or seasons may cause difference in BCCP values between populations and individuals (Bertelsen et al. 2007, Jones et al. 2014). BCCP reference values are however scarce in wildlife medicine. According to Jones et al. (2014) the use of index of individuality (IoI) can be useful to evaluate whether a population-based reference ranges are useful for detecting abnormal results in a single individual. Furthermore Jones et al. (2014) found out that there was a low degree of IoI in all biochemical variables for captive adult Bald eagles, hence these values can be used as guideline for other adult Bald eagles outside of the study population. Jones et al. (2014) also suggests that when species specific reference intervals are lacking, at least individual reference intervals should be established in order to use them as general guidelines.

2.5.1 Possible effects of Hg on blood clinical chemistry

There are only a few studies describing potential effects of Hg exposure in blood chemistry. However, studies describing possible effects of Hg contamination on haematology are far more common in birds, including white blood cell counts as well as other parameters, such as haematocrit (Whitney & Cristol 2018). Sonne et al. (2012) have investigated possible effects of organohalogen contaminants against BCCPs in WTEs in Norway, but studies evaluating possible effects of Hg in WTEs haven't been investigated before. High levels of Hg have been found to negatively affect normal hepatic function. Hoffman et al (2005) studied plasma chemistries between three groups of great egrets fed with Hg diet (control, low dose and high dose group). They found out decreased aspartate aminotransferase levels (AST) in accordance with increased Hg concentration in the blood. Other BCCPs which were affected significantly by Hg treatment included uric acid, total protein (TP), albumin and inorganic phosphorus. Also, calcium seemed to lower, but was not significant due to variability between tested individuals. In another study altered BCCPs indicating of liver damage, oxidative stress as well as possible kidney disturbance were found from young snowy egrets with high levels of Hg contamination (Hoffman et al. 2009). Significant Hg concentrations have also been found to affect blood mineral levels. Elbert & Anderson (1998) studied that blood potassium and phosphorus levels decreased with increasing brain tissue Hg concentration in western grebes. Significant decrease in blood calcium levels were noted after oral MeHg administration in domestic fowls

(Lundholm 1995). The relationship between Hg concentrations and triglyceride levels indicative of migration stopover performance were studied in another study. However, no correlation was found (Seewagen 2013).

2.6 The use of stable isotopes in avian ecology

Describing dietary habits are often required for conservational efforts, such as, understanding how dietary ecology relates to contaminant exposure in top predator species (Ramos et al. 2012). Large birds of preys are often described as generalist. According to a study by Nadjafzadeh et al. (2015) white-tailed eagles often optimize their dietary habits given the surrounding habitat and food availability. However local factors, such as food availability may cause a shift towards more specialized foraging strategies at the individual level (Nadjafzadeh et al. 2016). Several studies have also shown that temporal variation in the diet of WTEs occurs (Ekblad et al. 2016, Nadjafzadeh et al. 2016). Longitudinal diet studies are usually difficult to obtain, and conventional dietary analyses can underestimate certain prey types (Votier et al. 2003), therefore many studies have exploited stable isotope analyses in order to evaluate trophic level position as well as the sources of diet in relation to contaminant exposure (Eulaers et al. 2013, Nadjafzadeh et al. 2016).

Stable isotopes are nuclides of some of the known elements which do not decay spontaneously compared to radiogenic isotopes. The elements of interest for ecological studies are ones that occur in plants and animals, such as carbon and nitrogen (Ramos et al. 2012). There are slight differences in the mass of each isotope and usually the lighter stable isotope is more abundant in natural systems (Michener & Kaufman 2008). The ratios of heavy and light form of isotopes can furthermore be measured by using a mass spectrometer. These ratios are subtly affected during biological processes in a predictable manner, forming distinct isotopic signatures for each individual, target organ and habitat (Inger & Bearhop 2008). The latter process is called isotopic fractionation. The principal questions stable isotope studies try to explain are trophic positioning and the diet composition of an individual (Boecklen et al. 2011). Hence nitrogen, sulphur and carbon isotopes are usually used in dietary studies. Carbon is present in all three dietary macromolecules (protein, fat and carbohydrates) (Ramos et al. 2012). Carbon isotopes describes the geographical origin of dietary sources (marine vs. terrestrial) due to differences between the sources of carbon used for primary production (Kelly 2000, Marshall et al. 2007). Nitrogen isotopes can be used to access protein pathways, because nitrogen is present in protein compounds (Deniro & Epstein 1981). Moreover, nitrogen has traditionally been used to describe the trophic level in species diet, because $\delta^{15}N$ is enriched in consumers tissues, whereas $\delta^{14}N$ is excreted as nitrogenous waste products (Michener &

Kaufman 2008). This discrimination of nitrogen isotopes between the consumer and prey is usually described by trophic enrichment factors (TEFs) (Post 2002). For nitrogen isotopes TEFs varies typically between 2 and 4‰ at each trophic level (DeNiro & Epstein 1987, Peterson & Fry 1987) whilst the enrichment of carbon isotopes is lesser between trophic levels (Rounick & Winterbourn 1986, Peterson & Fry 1987). Additionally, it is recommended to generate isotopic baseline in order to evaluate the trophic level and the carbon flow of the consumer more accurately (Post 2002).

2.6.1 The use of feathers for evaluating stable isotope composition

Feathers can be used to assess isotopic patterns. The isotopic picture is dependent on the organs turnover time, for example isotopic measurement from plasma represents a relatively short period of dietary composition (Hobson & Clark 1993). Tissues which are cut off from the circulatory system after formation, such as hair and feathers, present the diet change over the formation period (Bond & Jones 2009). The lipid content of the feather may have an influence in $\delta^{13}C$ values, therefore lipid correction models (Boecklen et al. 2011) and lipid extraction methods have been described (Logan et al. 2008). According to Logan et al. (2008) the lipid extraction methods tends to affect stable isotopic values hence they were not used in this study.

3 MATERIALS AND METHODS

3.1 Field sampling

The present study was conducted on WTE nestlings and the sampling was performed in Finland at three different geographical areas: Varsinais-Suomi, Ostrobothnia and Lappi (Figure 1). Varsinais-Suomi is characterized by traditional agricultural land as well as by its coastal archipelago consisting of numerous islands. Two of the nests in Varsinais-Suomi were located on islands of the outer archipelago, surrounded by brackish water. Two of the nests were located inland close to fresh water lakes. The remaining nests in Varsinais-Suomi were located along the coast. The nest sites in Ostrobothnia were mainly located near the coast whereas the nest sites in Lapland were located inland around two big freshwater reservoirs (Lokka and Porttipahta). The collection of body feathers and blood was done during the breeding seasons of 2015 and 2016. Nests were monitored for breeding activity and successful nests were visited when nestlings were expected to be approximately 7 weeks old. During the breeding season of 2015 16 nestlings (belonging to 11 nests) were sampled while in the breading season of 2016

19 nestlings (belonging to 16 nests) were sampled. A detailed description of the study population characteristics is provided in Table 1.

Nestlings were taken from the nest for a minimal amount of time in order to minimise stress both to the nestlings and the parents. The nestlings were lowered from the nest in a nylon bags and sampled for blood and body feathers. Permission for tissue sampling was provided by the Finnish Animal Experimental Board and the competence of the sampler was confirmed by passing the accredited FELASA B course. From each nestling we collected 10 back feathers and a blood sample. Back feathers were collected by cutting them from the quill with scissors and were then stored in ziploc bags or paper envelopes at ambient temperature until chemical analysis. The puncture site was cleaned with disincentive (denatured ethanol) and 10 mL of blood was taken from the brachial vein with a heparinized needle (21 G) and a syringe to prevent clotting. The puncture site was pressed after needle withdrawal in order to avoid hematoma formation. The whole blood was consequently transferred from the syringe without a needle either to EDTA or lithium-heparin Vacuette® tubes and gently mixed to ensure even distribution of the anticoagulant in the blood sample. A remaining 1 mL of blood was transferred to an Eppendorf® tube. Blood samples were kept refrigerated prior to plasma separation at the end of each sampling day using a microcentrifuge (Ministar silverline, VWR) at 2000G. Plasma, whole blood and red blood cell samples were kept at -20°C until further chemical analysis.

Mass and morphological measurements were recorded concurrently with the sampling (Appendix 1). While the nestling was in the nylon bag, the weigh was measured with an electric hand balance with an accuracy of 50 g. Morphometrics were taken with a ruler and a sliding calliper (both ± 0.5 mm). In addition, recent feeding was judged by checking the crop size (empty, half-full, full). The age of the nestlings varied from 41 to 61 days old and was determined by employing the reported formula for age determination based on wing length (Helander 1981). The nestling's sex was determined by measuring the thickness of the tarsus at the thinnest point and using a cut-off value of 13.8 above which nestlings are assumed to be female (Helander et al. 2007).

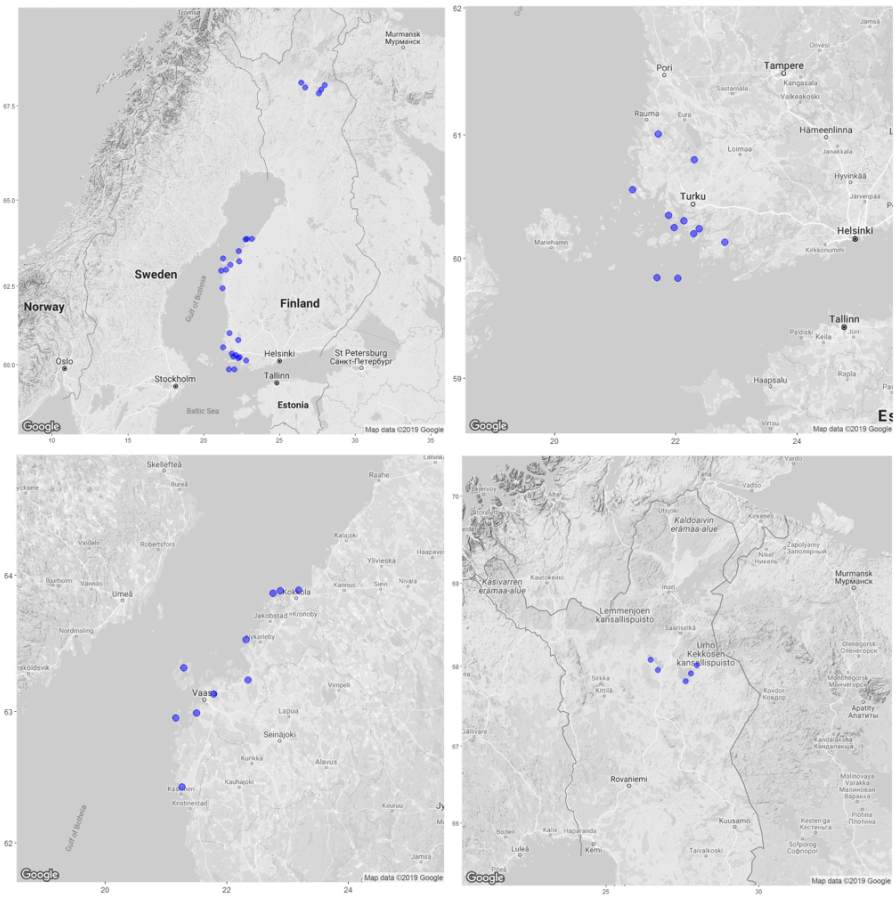

Figure 1. *Map identifying the study area and geographical distribution of sampled nests. Upper left panel: entire study region; upper right panel: Varsinais-Suomi study region; lower left panel: Ostrobothnia study region; lower right panel: Lapland study region.*

Table 1. *The study population properties, broken down per year and study region.*

	Varsinais-Suomi 2015	Varsinais-Suomi 2016	Ostrobothnia 2016	Lappi 2016
Nestlings	16	2	12	5
Nests	11	1	10	5
Brood size (mean and range)	1.7 (1-3)	3.0 (3)	1.5 (1-2)	1.4 (1-2)
Longitude (range)	59.83 - 61.01	60.13	62.43 - 63.89	67.68 - 68.08
Latitude (range)	21.29 - 22.39	22.80	21.16 - 23.18	26.46 - 27.99
Dates of sampling	05/06/2015 - 17/06/2015	27/05/2018 -27/05/2018	06/06/2016 - 04/07/2016	27/06/2016 - 30/06/2016

3.2 Chemical analysis of the feathers

The total mass and length of the body feathers were measured prior to washing and homogenisation (Appendix 2). All feathers were washed with milli-Q water and left overnight

to dry at ambient room temperature while covered with analytical paper. Subsequently, all feathers were cut in 1 mm pieces to create a homogeneous pool to be further subdivided for subsequent chemical analyses requiring specific sample treatment.

3.2.1 Stable isotope analysis

The stable nitrogen (N: ^{14}N and ^{15}N) and carbon (C: ^{12}C and ^{13}C) isotope composition of body feathers was carried out at the Centre for Permafrost at the University of Copenhagen, Denmark. A subsample of homogenized feather tissue was accurately weighed (1.50 ± 0.15 mg) into a tin combustion cup and was analysed for its isotopic composition using stable isotope ratio mass spectrometry (Finnigan MAT Delta PLUS, Thermo Scientific, Bremen, Germany) coupled in continuous flow to an elemental analyzer (CE 1110, Thermo Electron, Milan, Italy). The ratios of carbon and nitrogen stable isotopes are expressed as δ (‰), relative to the international standards Vienna PeeDee Belemnite (vPDB) and atmospheric N_2 (AIR), respectively. Calibration of the instrument was done using pure gases of CO_2 and N_2 against certified reference materials of sucrose and $(NH_4)_2SO_4$ provided by the International Atomic Energy Agency (IAEA, Vienna, Austria). The analytical procedure was positively assessed through procedural blanks, reference samples (Atropin) and analytical duplicate samples. Calculation of elemental data (N = 8.1 ± 0.1 % and C = 41.6 ± 0.1 %) was performed using Atropin. The analytical precision obtained was 0.1 ‰ SD, whereas a precision of 0.1 ‰ for $\delta^{13}C$ and of 0.2 ‰ for $\delta^{15}N$ were obtained.

3.2.2 Hg analysis

The analysis for total Hg (THg; hereafter referred to as Hg) was performed at the Trace Element Lab of the Department of Bioscience, Aarhus University, Roskilde, Denmark. Hg concentrations were determined using a Milestone DMA-80 Direct Hg Analyser (Sorisole, ITL) following the U.S. EPA Method 7473 (EPA, 1998). Prior to analysis, 10.5 mg ± 0.8 mg of dry homogenized feather material was weighed into a nickel boat and analysed by fixed wavelength atomic absorption spectrophotometry. The instrumental analytical quality control was verified by a procedural blank, duplicate samples, aqueous standards (10 ng and 100 ng Hg, prepared from 1000 ± 4 mg L^{-1} stock solution; Sigma-Aldrich, Switzerland), and standard reference material (SRM; DORM-4, National Research Council, Ottawa, Canada). Procedural blank and SRM samples were concurrently run every 10 samples. All samples were corrected for the average blank levels (0.06 ± 0.12 ng) as well as for the recovery of the aqueous standard (84.26% ± 34.03%). The resulting SRM values (0.41 ± 0.05 µg Hg g^{-1} dry weight) further

allowed assuring the reliability of the employed analytical procedure to determine feather Hg concentrations, expressed as μg g^{-1} dry weight (dw).

3.3 Blood clinical-chemical parameters

BCCP analyses were carried out at the Central Laboratory at the Department of Small Animal Clinical Sciences of the University of Copenhagen, Denmark. An automated spectrophotometric analyser containing ion-selective electrodes (ADVIA® 1800 Clinical Chemistry System, Munich, Germany. Siemens Healthcare GmbH) was used and all samples were analysed within 5 months after sampling All assays were subjected to daily internal and quarterly external quality controls. A standard blood clinical-chemical parameter package was chosen, comprising of 16 different parameters: Albumin (ALB; g L^{-1}), Glucose (GLU; mmol L^{-1}), Total protein (TP; g L^{-1}), Alkaline phosphatase (ALKP; U L^{-1}), Alanine aminotransferase (ALAT; U L^{-1}), Total bilirubin (Bil; μmol L^{-1}), Fructosamine (Fructo; μmol L^{-1}), Cholesterol (Cho; mmol L^{-1}), Creatinine (Crea; mmol L^{-1}), Inorganic phosphate (IP; mmol L^{-1}), Bile Acids (BA; mmol L^{-1}), Amylase (Amy; U L^{-1}), Urea (BUN; mmol L^{-1}), Gamma-glutamyltransferase (GGT; U L^{-1}), Sodium (Na; mmol L^{-1}) and Chloride (CL; mmol L^{-1}).

3.4 Statistical analysis

All statistical analyses were performed using R version 3.0.2 (R Development Core Team 2019) while rejecting the null-hypothesis at $\alpha = 0.05$. All model data and residuals were explored for influential outliers, normality and homoscedasticity (Zuur et al., 2010). The present study was aimed at investigating spatial trends and hence both sampling years' results for Varsinais-Suomi were as such pooled without explicit investigation for temporal variation in the parameters. The Hg data did not meet the requirement of being normally disturbed and was therefore ln-transformed.

Regional variation in $\delta^{13}C$ and $\delta^{15}N$ values and BCCP concentrations was investigated using one-way Analysis of Variance (ANOVA; *d13C ~ region*, *d15N ~ region* and *BCCP ~ region*). Regional variation in Hg concentrations was investigated by two-way ANOVA including each stable isotope value for normalisation (*log(Hg) ~ d13C*region* and *log(Hg) ~ d15C*region*). The relationship between $\delta^{13}C$ and $\delta^{15}N$ values was investigated using multiple linear regression including the regional effect on the relationship as well (*d15N ~ d13C*region*). Finally, the relationship between BCCP and Hg concentrations was investigated using simple linear regression (BCCP ~ log(Hg)). All post-hoc comparisons were made using Tukey's Honest Significant Differences and *P* values were adjusted for multiple comparison (P_{adj}).

4 RESULTS

4.1 Stable isotope values

4.1.1 Regional differences in $\delta^{13}C$ and $\delta^{15}N$ and the relationship between $\delta^{15}N$ and $\delta^{13}C$

$\delta^{13}C$ values were significantly different among the different regions ($F_{2,32}$=65.5; P<0.01). Pairwise comparisons showed that Varsinais-Suomi and Ostrobothnia were both significantly enriched in $\delta^{13}C$ values compared to Lappi (respectively +8.20‰ and +8.17‰; both P_{adj}<0.01). However, $\delta^{13}C$ values between Varsinais-Suomi and Ostrobothnia were not significantly different (P=0.99; Figure 2). Also, $\delta^{15}N$ values were significantly different among the different regions ($F_{2,32}$=6.44; P_{adj}<0.01). Pairwise comparisons showed that Varsinais-Suomi and Ostrobothnia were significantly enriched in $\delta^{15}N$ values compared to Lappi (respectively +2.34‰ and +2.35‰; both P_{adj}<0.01). However, $\delta^{15}N$ values between Varsinais-Suomi and Ostrobothnia were not significantly different (P_{adj}=0.99; Figure 2).

There was a significant relationship between $\delta^{15}N$ and $\delta^{13}C$ values ($F_{1,29}$=12.9; P<0.01), irrespective of the region ($F_{2,29}$=2.52, P=0.09). The insignificance of the effect of the region could be potentially due to low sample size and the lack of statistical power. The biplot analysis showed that stable isotope values cluster differently among populations, however there was one outlier of Varsinais-Suomi population showing a stable isotope composition distinct from other locations in that region (Figure 3 and 4).

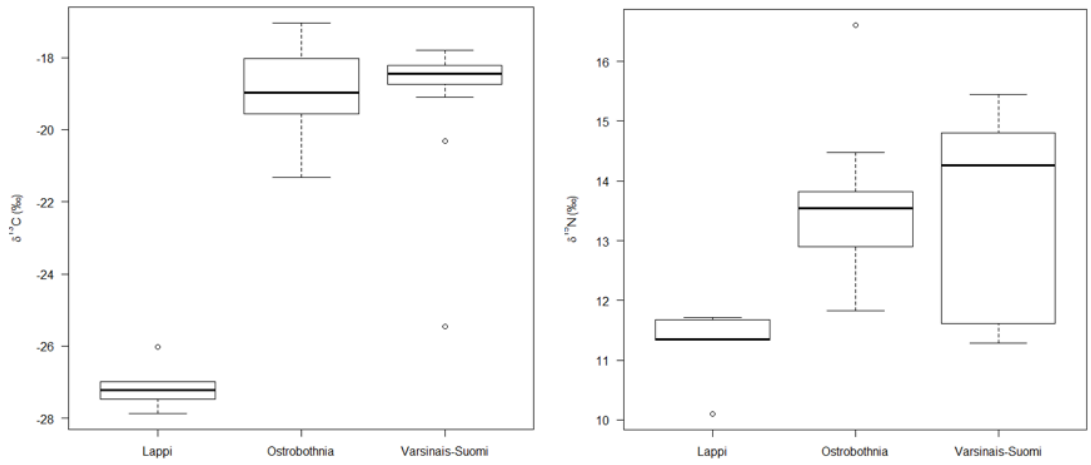

Figure 2. *Regional differences in stable isotope values ($\delta^{15}N$ and $\delta^{13}C$) according to the study populations. The upper and lower hinges represent the quartiles, the vertical lines represent*

the maximum and minimum data values and the bold line represents the median values. Points refers to potential outliers.

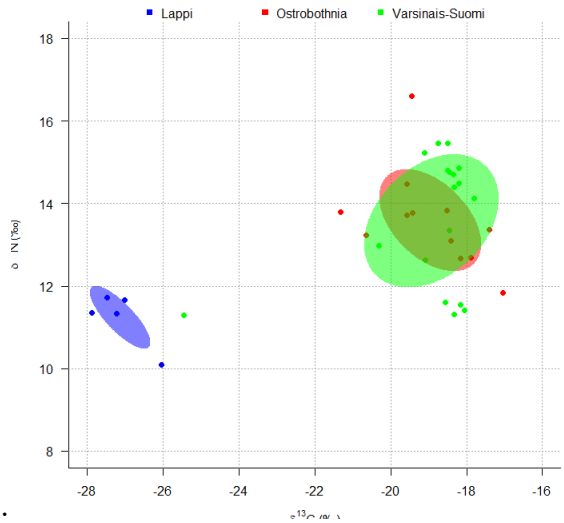

Figure 3. *Biplot analysis of $\delta^{15}N$ and $\delta^{13}C$ values in the feathers of WTE nestling in different populations. Lower left ellipse represents Lappi and the overlapping ellipses represent Ostrobothnia and Varsinais-Suomi.*

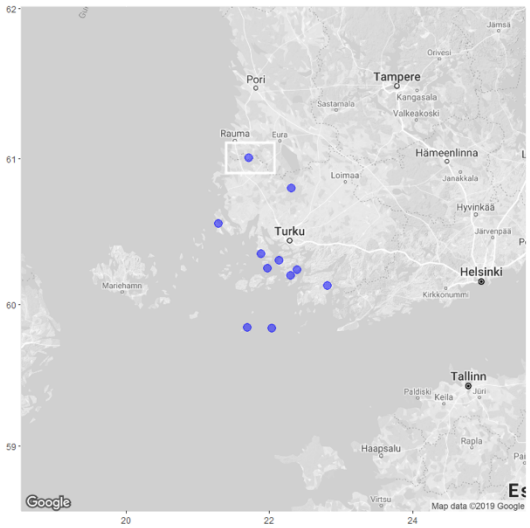

Figure 4. *Map identifying the location of a nest in Varsinais-Suomi with a distinct stable isotope composition.*

4.2 Hg

4.2.1 Regional differences in Hg

Hg values ranged from 2.48 µg g^{-1} to 13.10 µg g^{-1} and the greatest values were detected in Lapland (Figure 5; Table 2). Log-transformed Hg concentrations differed significantly among populations ($F_{1,32}$=27.18; P<0.01; Figure 6). Pairwise comparisons showed the Lappi population to have significantly higher Hg concentrations compared to those in Varsinais-Suomi and Ostrobothnia (both Padj<0.01). However, Hg concentrations were not significantly different between Ostrobothnia and Varsinais-Suomi (Padj=0.19; Figure 6). The highest Hg concentration (6,19 µg g-1) from Varsinais-Suomi was detected from the same nest showing a stable isotope composition distinct from other locations in that region (Figure 7).

4.2.2 Influence of dietary habits on variation in Hg contamination

There was a significant and negative relationship between δ15N values and Hg concentrations ($F_{1,29}$=37.99; P<0.01), irrespective of the region ($F_{2,29}$=0.30; P=0.74). There was also a significant positive relationship between $\delta^{13}C$ values and Hg concentrations ($F_{1,29}$=45.28; P<0.01), irrespective of the region ($F_{2,29}$=0.95; P<0.40).

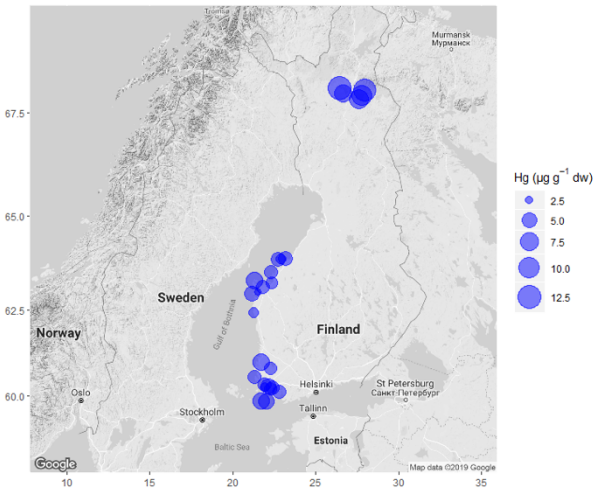

Figure 5. *Hg concentration (µg g^{-1}) among the study populations. Concentrations exceeding 5 µg g^{-1} have been reported to cause adverse effects on birds (Burger & Gochfeld 1997).*

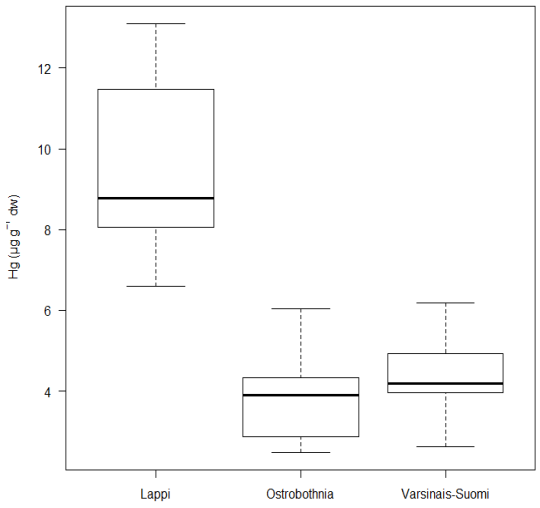

Figure 6. *Regional comparisons of Hg* concentrations µg g^{-1} for Lappi*, Ostrobothnia and Varsinais-Suomi populations. The upper and lower hinges represent the quartiles, the vertical lines represent the maximum and minimum data values and the bold line represents the median values.*

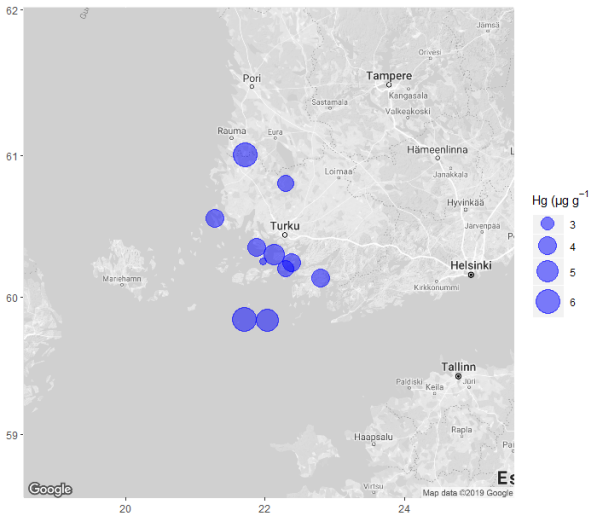

Figure 7. *Spatial variation of Hg concentrations in Varsinais-Suomi.*

Table 2. *Summary statistics [mean ± standard deviation (min-max)] for the Stable isotope values ($\delta^{13}C$ and $\delta^{15}N$) and Hg concentrations (Hg) in WTE nestlings from Varsinais-Suomi (2015-2016), Ostrobothnia (2016) and Lappi (2016).*

Study population	Varsinais-Suomi 2015 (n=16)	Varsinais-Suomi 2016 (n=2)	Ostrobothnia 2016 (n=12)	Lappi 2016 (n=5)
Stable isotope ecology (‰):				
$\delta^{13}C$	-19.25 ± 1.77 (-17.79 – (-25.45))	-18.62 ± 0.12 (-18.50 – (-18.74))	-18.98 ± 1.22 (-17.04 – (-21.32))	-27.07 ± 0.62 (-26.03 – (-27.87))
$\delta^{15}N$	13.33 ± 1.45 (11.29-15.22)	15.45 ± 0.003 (15.45 - 15.45)	13.68 ± 1.13 (11.83-16.61)	11.14 ± 0.59 (10.10-11.72)
Hg exposure (µg/g dw):				
	4.43 ± 0.96 (2.62-6.19)	4.29 ± 0.46 (3.97-4.61)	3.86 ± 1.02 (2.48 - 6.04)	9.67 ± (6.59-13.10)

4.3 BCCPs

4.3.1 Regional differences in BCCPs

There were significant regional differences for the following BCCPs: albumin, total protein, alkaline phosphate, alanine aminotransferase, fructosamine, creatinine, bile acids, amylase, gamma-glutamyl transferase, natrium, glucose, urea and chloride ($3.64 \leq F_{2,32} \leq 17.76$; $0.01 \leq P \leq 0.04$; Figure 8; Table 3). Pairwise comparisons showed the following BCCPs to be significantly higher in Lappi compared to Varsinais-Suomi: albumin, total protein, alanine aminotransferase, fructosamine, creatinine, bile acids, amylase, gamma-glutamyl transferase and glucose ($0.01 < P_{adj} < 0.05$). The following BCCPs were also significantly higher in Ostrobothnia compared to Varsinais-Suomi: albumin, total protein, alkaline phosphatase, glucose and urea ($0.01 < P_{adj} \leq 0.05$). Alanine aminotransferase and creatinine were also significantly higher in Lappi compared to Ostrobothnia (respectively $P_{adj} < 0.01$ and $P_{adj} = 0.039$).

There were no significant regional differences for the following BCCPs: total bilirubin, cholesterol and inorganic phosphate ($0.51 \leq F_{2,32} \leq 1.58$; $0.22 \leq P \leq 0.60$). Furthermore, pairwise comparisons failed to show a significant difference between Ostrobothnia and Lappi for the following BCCPs: albumin, total protein, alkaline phosphatase, total bilirubin, fructose, cholesterol, creatinine, inorganic phosphate, bile acids, amylase, gamma-glutamyl transferase, natrium, glucose, urea or chloride ($0.12 \leq P_{adj} \leq 0.99$). There was neither a significant difference between Varsinais-Suomi and Lappi concentrations of the following BCCPs: alkaline phosphatase, total bilirubin, cholesterol, inorganic phosphate, natrium, urea or chloride ($0.1 < P_{adj} \leq 0.99$). There was neither a significant difference between Varsinais-Suomi and Ostrobothnia for the following BCCPs: alanine aminotransferase, total bilirubin, fructosamine,

cholesterol, creatinine, inorganic phosphate, bile acids, amylase, gamma-glutamyl transferase, natrium or chloride ($0.13 \leq P_{adj} \leq 0.74$).

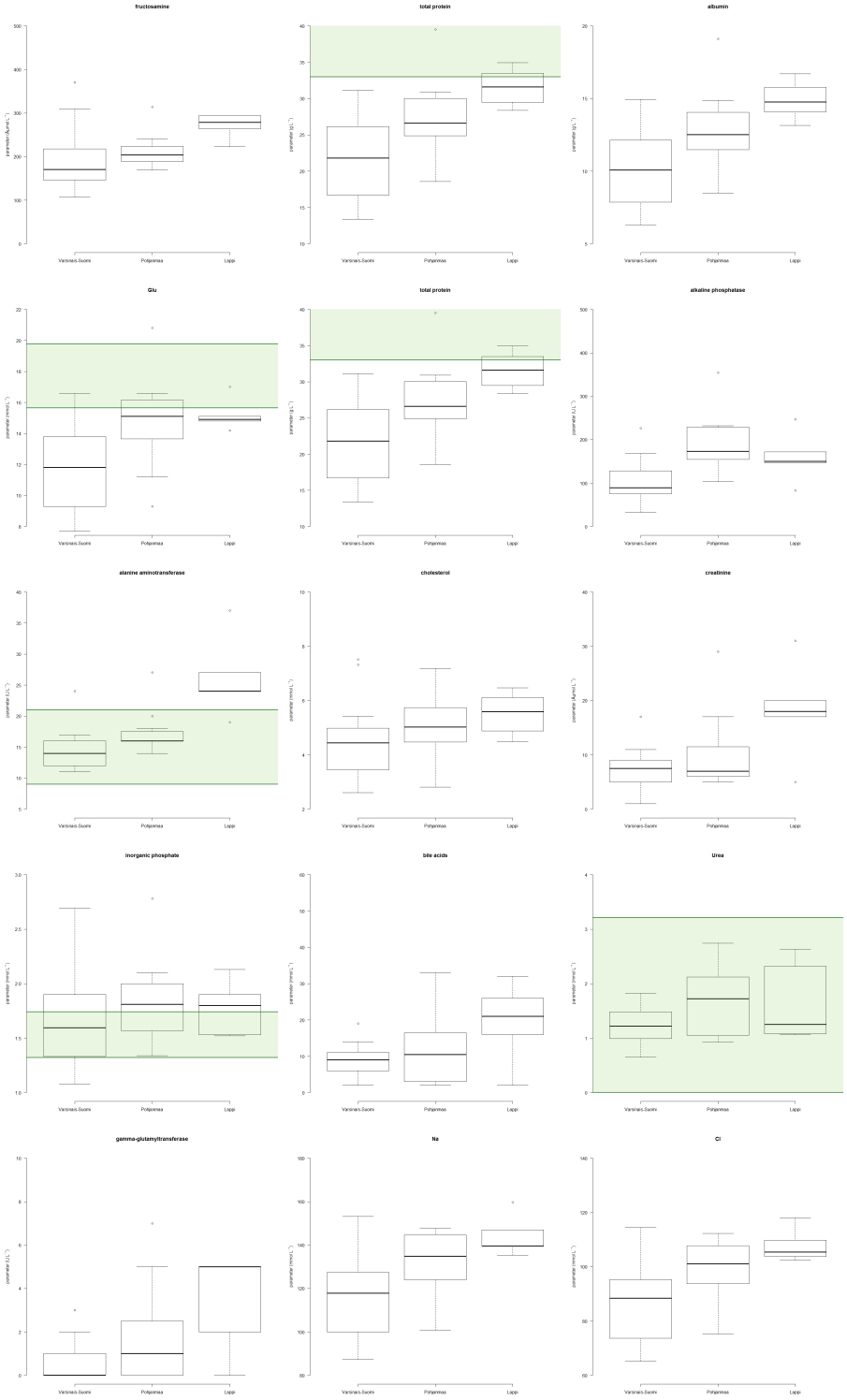

Figure 8. *Differences in BCCPs among regions. The upper and lower hinges represent the quartiles, the vertical lines represent the maximum and minimum data values and the bold line*

represents the median values. Green boxes showing reference ranges for Bald Eagles according to Mealey et al. (2004) (Units transferred from US to IS).

Table 3. *Summary statistics [mean ± standard deviation (min-max)] of BCCPs in WTE nestlings from Varsinais-Suomi (2015-2016), Ostrobothnia (2016) and Lappi (2016).*

Study population	Varsinais-Suomi 2015 (n=16)	Varsinais-Suomi 2016 (n=2)	Ostrobothnia 2016 (n=12)	Lappi 2016 (n=5)
Alkaline phosphatase (U L^{-1})	97.12 ± 40.23 (33.0 - 168.0)	161 ± 65 (96.0 - 226.0)	199.36 ± 61.55 (103.0 - 354.0)	159.80 ± 52.75 (83.0 - 247.0)
Albumin (g L^{-1})	10.31 ± 2.63 (6.31 - 14.94)	12.51 ± 2.32 (10.19 - 14.83)	13.03 ± 2.49 (8.50 - 19.11)	14.90 ± 1.25 (13.15 - 16.70)
Urea (mmol L^{-1})	1.15 ± 0.30 (0.65 - 1.65)	1.69 ± 0.14 (1.55 - 1.82)	1.70 ± 0.58 (0.93 - 2.74)	1.67 ± 0.67 (1.06 - 2.63)
Cholesterol (mmol L^{-1})	4.61 ± 1.28 (2.61 - 7.52)	4.44 ± 0.99 (3.45 - 5.42)	5.08 ± 1.12 (2.81 - 7.18)	5.50 ± 0.74 (4.49 - 6.47)
Fructosamine (µmol L^{-1})	192.0 ± 68.3 (107.0 - 370.0)	216.0 ± 59.0 (157.0 - 275.0)	216.07 ± 37.4 (169.0 - 314.0)	267.29 ± 26.36 (223.0 - 294.0)
Creatinine (µmol L^{-1})	7.0 ± 2.52 (1.00 - 11.0)	11.5 ± 5.50 (6.00 - 17.0)	11.14 ± 6.61 (5.00 - 29.0)	18.14 ± 8.28 (5.0 - 31.0)
Total Bilirubin (g L^{-1})	11.53 ± 6.07 (5.10 - 29.10)	5.10 ± 0.50 (4.60 - 5.60)	8.55 ± 3.77 (3.90 - 15.80)	10.18 ± 2.55 (7.30 - 14.0)
Alanine aminotransferase (U L^{-1})	14.78 ± 3.33 (11.0 - 24.0)	14.5 ± 1.50 (13.0 - 16.0)	17.79 ± 3.25 (14.0 - 27.0)	26.71 ± 5.98 (19.0 - 37.0)
Gamma-glutamyltransferase (U L^{-1})	0.39 ± 0.58 (0.00 - 2.0)	1.5 ± 1.50 (0.00 - 3.0)	1.93 ± 2.17 (0.00 - 7.0)	3.14 ± 2.1 (0.00 - 5.0)
Amylase (U L^{-1})	13.72 ± 7.02 (7.0 - 35.0)	35.0 ± 15.2 (18.0 - 52.0)	31.86 ± 17.0 (14.0 - 63.0)	49.29 ± 30.0 (20.0 - 100.0)
Total protein (g L^{-1})	21.9 ± 5.41 (13.41 - 31.10)	25.63 ± 4.27 (21.36 - 29.9)	27.69 ± 4.90 (18.57 - 39.50)	31.61 ± 2.44 (28.39 - 34.96)
Bile acids (mmol L^{-1})	8.61 ± 3.27 (2.00 - 14.0)	13.50 ± 5.50 (8.00 - 19.0)	12.14 ± 8.63 (2.00 - 33.0)	18.71 ± 10.19 (2.00 - 32.0)
Glucose (mmol L^{-1})	11.72 ± 2.53 (7.70 - 16.50)	13.90 ± 2.70 (11.20 - 16.60)	14.83 ± 2.74 (9.30 - 20.80)	15.31 ± 0.95 (14.20 - 17.00)
Inorganic phosphate (mmol L^{-1})	1.64 ± 0.40 (1.08 - 2.69)	1.94 ± 0.145 (1.79 - 2.08)	1.86 ± 0.37 (1.34 - 2.78)	1.79 ± 0.23 (1.52 - 2.13)
Sodium (mmol L^{-1})	117.74 ± 19.18 (87.40 - 150.2)	133.20 ± 20.0 (113.20 - 153.2)	138.89 ± 23.08 (100.8 - 200.0)	145.17 ± 8.60 (135.4 - 159.7)
Chloride (mmol L^{-1})	88.08 ± 14.73 (65.27 - 114.45)	99.28 ± 14.03 (85.25 - 113.31)	104.97 ± 18.29 (75.34 - 154.51)	107.92 ± 5.625 (102.47 - 118.02)

4.3.2 Relationship between BCCPs and Hg

Hg exposure was positively associated to levels of alanine aminotransferase ($F_{1,33}$=9.58; P<0.01) and total bilirubin ($F_{1,33}$=6.51; P=0.02). There were no significant relationships between Hg exposure and the remaining BCCPs ($0.05 \leq F_{1,33} \leq 1.89$; $0.15 \leq P \leq 0.57$).

4.3.3 Comparison of BCCP values from different studies

The BCCP values from three different sub-populations in Finland showed similar values when compared to BCCP results reported in another population of WTE nestlings in Norway (Sonne et al. 2012), which used the same laboratory as was used in the present study. Additionally, study conducted in bald eagle (*Haliaeetus leucocephalus*) nestlings studied in the USA

(Mealey et al. 2004; Bowerman et al. 2002), showed similar results for most of the parameters (table 4). There were however some parameters showing inconsistent results: alkaline phosphatase, urea, total bilirubin and amylase. Also, some of the parameters were lacking in some studies.

Table 4. *Total BCCP values (mean ± SD [min-max]) from bald eagles (Mealey et al. 2004 and Bowerman et al. 2002) and white-tailed eagles (Sonne et al. 2012). Units have been converted from US to SI.*

BCCPs	White-tailed eagle Finland	Bald eagle USA[1]	Bald eagle USA[2]	White-tailed eagle*, Northern Norway[3]
number of samples tested (n)	**n = 35**	**n = 59-151**	**n = 51**	**n = 15**
Alkaline phosphatase (U L-1)	142.4 ± 67.63 (33.0-35)	147.57 ± 51.96 (35.2-322.0)	449 ± 91.7 (295 - 654)	1192 (415–1921)
Albumin (g L-1)	11.96 ± 2.95 (6.31 - 19.11)	14.8 ± 0.43 (10.0-32.0)	14.0 ± 2.0 (10 - 18)	14.3 (10.4–17.2)
Urea (mmol L-1)	1.19 ± 0.54 (0.65 - 1.65)	4.79 ± 0,51 (0.71 - 15.7)	1.642 ± 0.57 (0.53 - 2.856)	1.6 (0.85–2.5)
Cholesterol (mmol L-1)	4.38 ± 1.20 (2.61 - 7.32)	-	5.485 ± 0.84 (3.367 - 7.93)	6.48 (5.26–9.47)
Fructosamine (µmol L-1)	186.21 ± 63.29 (107 - 370)	-	-	203 (161–253)
Creatinine (µmol L-1)	7.14 ± 6.55 (1.00 - 11.00)	1.268 ± 5.57 (0.47-31.90)	21.57 ± 0.60 (10.17 - 32.90)	11.6 (6–31)
Total Bilirubin (g L-1)	9.28 ± 5.00 (5.10 - 17.30)	124 ± 106 (30 - 410)	23 ± 17 (5 - 70)	14.57 (9.5–28.2)
Alanine aminotransferase (U L-1)	14.29 ± 5.424 (11.00 - 24.00)	17.11 ± 7.41 (3.0 - 42.0)	15.5 ± 6.7 (2 - 34)	20.5 (16–27)
Gamma-glutamyltransferase (U L-1)	0.21 ± 1.91 (0.00 - 2.00)	-	-	2.5 (1–6)
Amylase (U L-1)	12.79 ± 20.21 (7.00 - 35.00)	-	684.7 ± 248.7 (324 - 1357)	466 (182–801)
Total protein (g L-1)	21.39 ± 6.01 (13.41 - 31.10)	32.8 ± 9.8 (14.0 - 104)	34 ± 5 (25.0 - 55.0)	30.2 (22–42.6)
Bile acids (mmol L-1)	9.00 ± 7.73 (2.00 - 14.00)	-	-	22.33 (2–40)
Glucose (mmol L-1)	11.35 ± 2.92 (7.7 - 16.50)	12.39 ± 1.52 (8.25 - 16.2)	15.4 ± 1.79 (5.28 - 18.70)	14.25 (11.93–15.97)
Inorganic phosphate (mmol L-1)	1.52 ± 7.73 (2.0 - 14.00)	2.0 ± 0.42 (1.0 - 3.682)	1.938 ± 0.226 (3.0 - 3.811)	2.07 (1.48–3.17)
Sodium (mmol L-1)	115.26 ± 22.35 (87.40 - 149.30)	104.98 ± 10.11 (67.00 - 127.00)	148.0 ± 2.3 (143.2 - 153.3)	151 (133–159)
Chloride (mmol L-1)	86.19 ± 17.29 (65.27 - 114.45)	104.98 ± 10.11 (67.0-127.0)	117.0 ± 2.5 (111.7 - 121.8)	-

1. *Mealey et al. 2004; 2. Bowerman et al. 2002; 3. Sonne et al. 2012.*

5 DISCUSSION

5.1 Regional differences in stable isotope values

A significant difference in $\delta^{13}C$ and $\delta^{15}N$ values in Varsinais-Suomi and Ostrobothnia compared to Lappi was apparent and is likely to indicate variable local dietary habits. Nonetheless, the concurrent variation in $\delta^{15}N$ and $\delta^{13}C$ values is indicative of changes in prey composition rather than regional changes in baseline isotope signatures (Eulaers et al. 2014). Although we could not standardize baseline values for $\delta^{15}N$ (Post 2002), a larger range in $\delta^{15}N$ values in Varsinais-Suomi and Ostrobothnia can be observed compared to Lappi. This may suggest a greater degree of trophic diversity in the food chain of WTEs from Varsinais-Suomi and Ostrobothnia (Layman et al. 2007). Moreover, literature suggests that eagles can have a broad diet in coastal regions (Elliot et al. 2009). Varsinais-Suomi showed also greater variance in $\delta^{13}C$ values compared to Ostrobothnia and Lappi, which can reflect greater number of basal carbon resources (Layman et al. 2007). Carbon isotopes can be used to trace different carbon origins to a consumer. For example, $\delta^{13}C$ values from terrestrial plants tends to be depleted compared to marine algae, and these differences can persist throughout the food chain (Kelly 2000, Marshall et al. 2007). Our results show significant depletion of $\delta^{13}C$ values in Lappi compared to Varsinais-Suomi and Ostrobothnia. This spatial variability in $\delta^{13}C$ values could discriminate coastal versus freshwater feeding between the coastal and inland populations (France 1995, Bearhop et al. 1999). Elliot et al. (2009) reported similar isotopic enrichment patterns, showing depletion of $\delta^{13}C$ values in Bald eagles feeding in fresh water compared to those feeding in marine inshore and offshore sites. Although stable isotope values from only the WTE cannot provide detailed information on prey species composition (Post 2002) our results are consistent with previous dietary studies done on Finnish WTEs which have shown significant regional differences in prey species from three different WTE populations in Finland (Sulkava et al. 1997). According to Sulkava et al. (1997) the main prey for the Lappi population is Northern perch (*Esox lucius*) while the proportion of birds were greater in Southwest Finland and the Quark region.

As marine organisms are considered to be enriched in $\delta^{15}N$ values compared to terrestrial species, the finding is consistent with the fact that coastal WTE populations are feeding on marine species (Schoeninger & DeNiro 1984). However agricultural use of nitrogen fertilizers may partially account for enriched $\delta^{15}N$ values in Varsinais-Suomi and Ostrobothnia (Vander Zanden et al. 2005, Hofmeister et al. 2013). This possible confounding factor could not be investigated in the present study. The significant difference in $\delta^{15}N$ values between coastal and Lappi populations could be explained by different baseline values for $\delta^{15}N$ or

changes in the food web structure. However, without standardizing the baseline values this difference cannot be pointed out (Post 2002).

The coordinates for the outlier from Varsinais-Suomi population, which was showing a stable isotope value distinct from other locations in that region, show that this nest was located more inland compared to other nest sites (distance from the coast <15km) and it was surrounded by numerous smaller and one bigger lake. This finding contributes to valuable use of stable isotope analysis to study intraspecific variation in dietary specialisation (Bolnick et al. 2003).

5.2 Regional differences in Hg concentration and the effect of dietary habits

Our data show that the Lappi population had significantly higher Hg concentrations compared to the ones in Varsinais-Suomi and Ostrobothnia, exceeding the concentration of 5 μg g^{-1} which have been reported to cause adverse effects on birds (Burger & Gochfeld 1997). These levels are similar that have been found from the mantle feathers of juvenile fish-eating birds of prey, such as Osprey (*Pandion haliaetus*) from Finland in the 1972-1980 (Häkkinen & Häsänen 1980) and 1984-1987 (Solonen & Lodenius 1990). However, even with a feather concentration of 40 μg g^{-1} and 66 μg g^{-1} in bald eagles (*Haliaeetus leucocephalus*) did not show any adverse effects (Bowerman et al. 1994, Weech et al. 2009). Mean values of Hg in Varsinais-Suomi and Ostrobothnia (4,41 and 4,29 μg g^{-1} respectively) fell below the reported limit concentration of 5 μg g^{-1} by Burger & Gochfeld (1997). However, four individuals from Varsinais-Suomi exceeded this threshold.

Since there was a high correlation between Hg and stable isotope values, dietary habits seem to have a strong effect on Hg exposure in WTE nestlings in Finland, although the interaction effect of the region didn't explain the relationship between isotope values and Hg exposure. There was a significant negative relationship between δ^{15}N value and Hg exposure. This negative trend seems odd, because elevated Hg concentrations are usually related to higher δ^{15}N values (Atwell et al. 1998). However, we cannot determine whether the significantly lower δ^{15}N values in Lapland are actually due to lower trophic positioning of WTEs compared to Ostrobothnia and Varsinais-Suomi since we couldn't standardize baseline δ^{15}N values in the present study. Furthermore, it is known that δ^{15}N values are generally lower in freshwater ecosystems compared to marine ones (France 1994). Significant difference of Hg exposure in Lappi compared to coastal populations could be therefore explained by locally elevated Hg levels in the environment or bioaccumulation and biomagnification of mercury in the organisms and food webs (Watras et al. 1998, Miller et al. 2013). Furthermore, methylation of Hg occurs more readily in freshwater environments (Gilmour et al. 2013). Locally elevated Hg concentrations might be due to increased atmospheric deposition, although decrease in the

atmospheric Hg has been noted (Zhang et al. 2016). Locally elevated Hg concentrations could also be due to discharges from mines and other local sources and can therefore increase the Hg concentration in primary and secondary consumers, such as in fish (Painter et al. 2016, Suchanek et al. 2009). It is also known that artificial reservoirs, such as Porttipahta and Lokka, are usually more problematic considering Hg concentrations due to increased Hg runoff from surrounding soil (Morel et al. 1998, Dittman & Driscoll 2009). In Finland Hg levels in fish have been monitored due to local environmental programmes as well as government led assessments (Mehtälä 2014, Airaksinen et al. 2018). Although Hg concentrations have been decreased notably in the lakes since 1970 (Verta et al. 2010) studies conducted in Lokka and Porttipahta have shown that increased Hg concentrations can occur in larger fish individuals (Mehtälä 2014). According to Nadjafzadeh et al. (2015) WTEs also select towards larger fish when the prey is abundant in order to optimize energy consumption and prey profitability. Furthermore, it has been shown that piscivorous species are at greater risk of MeHg exposure (Scheuhammer 2007). Since earlier studies show that the main prey for WTEs in Lappi is fish, it can be hypothesized that this could drive Hg exposure and partially explain elevated Hg concentrations in the feathers of the Lappi WTE population. Other potential factors related to elevated Hg concentrations in Lappi WTEs could be age and body size, however these biological variables where not taken into account in the present study.

Studies conducted in Bald eagle nestlings in Virginia, USA, found similar Hg concentration, approaching sub-lethal levels, in the feathers of inland population compared to our findings in Lapland (mean Hg concentration in feathers 8.43 μg g^{-1}) (Desorbo & Evers 2007, Kramar et al. 2019). It has also been suggested that the most susceptible time period to exceed blood Hg threshold levels would be at the time of fledging due to withered blood supply into feathers (Condon et Cristol 2009). These findings raise concerns of possible health effects in fledged nestlings which are exposed to increased Hg concentrations in Lappi.

5.3 Regional differences in BCCPs and the effect of Hg concentration

Our results showed great variance of BCCPs among the different study regions. Nine out of 16 studied parameters were significantly higher in Lapland compared to Varsinais-Suomi. Also 5 out of 16 parameters were significantly higher in Ostrobothnia compared to Varsinais-Suomi. Only alanine aminotransferase and creatinine were higher in Lappi compared to Ostrobothnia. Since the fit of the model for the relationship between BCCPs and region were mostly moderate, these changes are probably influenced by other factors such as age, diet, feeding state or sex ratio between populations. However, the effect of these biological traits was not

studied in the present study due to inaccuracy and the partial lack of morphometric measurements which could affect the estimates of the age and sex.

Our results showed significant increases in alanine aminotransferase and total bilirubin values with increased Hg concentrations. Whereas alanine aminotransferase is highly liver-specific in most mammals (Thrall et al. 2004), in birds it is present in numerous tissues, such as liver, kidney as well as in skeletal muscles (Lumeij & Westerhof 1987, Joseph 1999). Hence, elevated levels are difficult to interpret. Possible reported factors causing elevated alanine aminotransferase values are aging (Gylstorff & Grimm 1987) and seasonal variation, independent of reproduction activity (Gerlach et al. 1979). Increased activities could also occur due to pathological changes in various tissues (Lumeij & Westerhof 1987). Bilirubin is the metabolic breakdown product of heme and increased bilirubin has been associated with cholestasis as well as liver failure. Elevated total bilirubin values could be therefore explained by negative impact on liver function, but diagnostic sensitivity and specificity of total bilirubin should be further assessed (Harrison & Lightfoot 2005). Previous studies have found high Hg concentration to possibly affect liver and kidney biochemistries (Hoffman et al. 2005, Hoffman et al. 2009), although impacted parameters were different compared to the present study. Furthermore, since the present study cannot differentiate the possible confounding effects and on-going environmental variables, we could not pinpoint any clear cause-and-effect relationships.

6 CONCLUSIONS AND RECOMMENDATIONS

The results of the present study indicate for distinct feeding niches between different WTE populations in Finland. Strong correlations between feather Hg concentrations and stable isotope values show dietary habits to drive intraspecific variability in Hg exposure. Future studies should focus on modelling individual diet composition, which could reveal more specific understanding on the mechanism of contaminant exposure. The underlying factors causing significantly higher levels of Hg in Lappi compared to Ostrobothnia and Varsinais-Suomi should be also assessed in further studies. Our results suggest a possible impact of Hg exposure on the liver function, indicated by elevated total bilirubin values. Biological variables should be taken into account in statistical analyses, since elevation of alanine aminotransferase and total bilirubin could also be explained by these factors. Further studies should additionally investigate possible significance of alanine aminotransferase activity in raptors. Since sensitivity to Hg exposure is species specific regarding different end-points, further studies should investigate whether these Hg concentrations are ecologically relevant in the study

populations and whether possible impacted liver function by elevated Hg levels has any significant effect on the health of WTE nestlings. Moreover, in order to use BCCPs as general guidelines, reference intervals for WTE eagle nestlings in Scandinavia should be created. This would acquire consistent sampling for a longer period of time. Long-term sampling programmes would also serve for monitoring pollutant trends. The accuracy in gathering morphometric measurements is also crucial in order to use biological traits as explanatory factors in statistical models. In addition, studies finding correlation between morphometric measurements and age or sex should be repeated to ensure their usefulness in particular study populations.

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9 APPENDICES

Appendix 1. *Table showing each morphological measurement per studied individual. Abbrevations for following measurements are beak length (beak.l), beak hight (beak.h), tarsus depth (tarsus.d), tarsus width (tarsus.w), tarsus lenght (tarsus.l), wing lenght (wing.l), respectively. Dashes stand for lacking measurements.*

ID.field	age	brood	beak.l	beak.h	tarsus.d	tarsus.w	tarsus.l	wing.l	mass	crop	crop.mass
E21477	50	2	-	-	12.7	16.3	-	400	3960	-	-
E21476	52	2	-	-	14.8	17.6	-	420	4680	-	-
E22742	53	1	-	-	12.5	14.5	-	430	3400	-	-
E22741	47	2	-	-	12.9	15.2	-	375	4140	-	-
E22740	49	2	-	-	12.7	15.4	-	390	4000	-	-
E26131	45	1	-	-	14.0	16.1	-	360	3780	-	-
E26080	53	2	-	-	15.4	17.0	-	425	4800	-	-
E26081	51	2	-	-	14.6	16.7	-	410	4700	-	-
E22958	53	1	-	-	13.7	15.8	-	425	4300	-	-
E22739	54	2	-	-	14.7	17.1	-	440	5200	-	-
E22738	58	2	-	-	15.8	17.8	-	475	5540	-	-
E26195	49	1	-	-	13.9	15.9	-	395	4340	-	-
E22717	52	3	-	-	14.5	17.3	-	420	5080	-	-
E22718	51	3	-	-	13.0	16.3	-	415	4180	-	-
E22655	48	2	-	-	13.0	15.4	-	380	3780	-	-
E22654	53	2	-	-	15.8	17.3	-	425	4900	-	-
E23967	55	3	-	-	-	-	-	445	4240	yes	100
E23969	47	3	-	-	-	-	-	490	5140	no	0
E22882	49	1	-	-	-	-	-	395	5220	yes	50
E22880	50	1	46.0	34.0	13.0	16.0	85.0	400	4280	yes	50
E22877	48	1	47.0	43.5	14.5	17.0	101.0	385	4480	yes	50
E22888	51	2	50.0	34.0	16.0	17.5	84.0	410	4690	no	0
E26859	55	1	49.0	31.0	16.0	18.0	93.0	445	4240	no	0
E22800	55	2	49.0	31.5	13.0	15.0	83.0	450	4080	no	0
E22799	55	2	52.0	35.0	15.0	18.0	92.5	450	5200	no	0
E22789	52	1	54.0	31.0	15.0	17.5	98.0	420	4640	no	0
E22883	54	2	51.0	35.0	14.0	18.0	101.0	435	4800	no	0
E22884	48	2	50.0	35.0	15.0	17.5	98.5	385	4440	no	0
E22845	58	2	44.5	31.5	17.0	19.0	99.0	475	4300	yes	50
E22090	47	1	47.0	32.5	14.0	15.5	92.0	375	4780	yes	50
E22091	61	1	47.5	33.0	15.0	17.0	97.0	505	4900	yes	100
E22096	58	1	47.0	32.5	13.0	15.0	97.0	475	3880	yes	50
E22104	50	2	49.0	33.5	14.5	17.0	99.0	400	5000	yes	50
E22113	48	2	45.0	31.0	14.0	17.0	92.0	380	3360	no	0
E26805	50	2	46.5	34.0	15.0	17.0	100.0	400	4340	no	0

Appendix 2. *Table showing the mass (g) and total length (mm) of the feathers per individual.*

Study ID	Mass (g)		Total length (mm)
	feathers with package	feathers withouth package	
E21477	3.18	0.41	73
E21476	3.17	0.62	57
E22742	2.97	0.56	94
E22741	2.88	0.45	52
E22740	2.72	0.43	58
E26131	2.96	0.44	54
E26080	2.99	0.49	69
E26081	3.08	0.56	63
E22958	2.92	0.64	57
E22739	3.15	0.61	67
E22738	3.39	0.92	73
E26195	3.15	0.52	55
E22717	3.07	0.55	71
E22718	3.40	0.87	52
E22655	2.93	0.44	64
E22654	3.02	0.58	62
E23967	4.61	0.52	131.7
E23969	4.78	0.66	141.6
E22882	4.10	0.51	115.6
E22888	3.55	0.8	129.1
E22845	3.19	0.33	66.47
E22789	3.42	0.67	129.9
E22800	3.51	0.74	150.7
E22884	3.35	0.59	108.2
E22880	3.27	0.44	102.7
E22799	4.33	0.74	132.2
E22877	3.40	0.57	104.9
E26805	4.18	0.56	110.1
E26859	4.41	0.68	132.8
E22091	3.45	0.58	88
E22096	4.24	0.49	101.9
E22883	3.25	0.37	79
E22113	3.15	0.35	84.5
E22104	3.26	0.42	82
E22090	3.22	0.34	76.7
E22098	3.29	0.45	96.8